turning knowledge into practice

Research Triangle Park, North Carolina

One-Generation Extension Study of Vinclozolin and Di-n-Butyl Phthalate Administered by Gavage on Gestational Day 6 to Postnatal Day 20 in CD® (Sprague-Dawley) Rats

L. Earl Gray, Jr., Ph.D. (EPA)

Paul M. D. Foster, Ph.D. (NIEHS)

Eric A. Crecelius, Ph.D. (Battelle)

Rochelle W. Tyl, Ph.D (RTI International)

Julia D. George, Ph.D. (RTI International)

RTI INTERNATIONAL

BACKGROUND

This study was performed under a subcontract to Battelle Laboratories, Columbus, Ohio for prime contract No. 68-W-01-023 for the U.S. Environmental Protection Agency

One of the tests being considered for inclusion in this screening program is a mammalian, two-generation reproductive toxicity test EPA OPPTS Health Effects Test Guideline 870.3800: Reproduction and Fertility Effects (U.S. EPA 1998).

Although the basic two-generation study design was developed to provide information on insult to the reproductive tract, there is concern that certain effects may be missed, simply because the reproductive tract has not had sufficient time to develop before the observations are made.

In the standard two-generation test, most F1 animals are sacrificed and examined at postnatal day (pnd) 21; only one animal per sex per litter is usually allowed to continue to maturity. These animals are used to breed the F2 generation.

The study design being tested through this work assignment examined whether or not allowing more of the F1 generation males to continue through puberty to adulthood will provide additional information in detecting endocrine-mediated effects.

OBJECTIVES

1. **Whether some of the effects from perinatal exposure to Vinclozolin (VIN) or to Di-n-butyl phthalate (DBP) that can be easily detected after puberty <u>are missed</u> in weanling animals of the F1 generation.**

2. **Whether some of these effects occur at an incidence that would go undetected if only one male per litter is retained past puberty and examined at adulthood.**

HYPOTHESIS

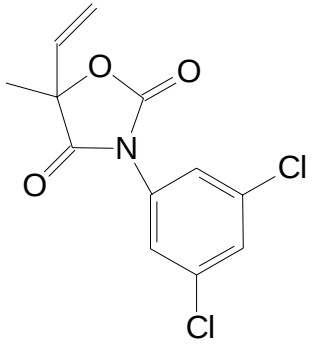
- **The "Standard 2-Generation Protocol" cursory examination of up to three F1 males per litter at weaning and only one F1 male at adulthood allows adverse reproductive effects that appear at and after puberty to be missed**

- **Examination of three or more F1 males at or after puberty, in addition to the F1 males examined at weaning, will detect additional reproductive effects, and provide a more complete and accurate characterization of the effects of the test compound**

APPROACH

- **Vinclozolin (VIN) and dibutyl phthalate (DBP), two known and well-characterized anti-androgens, were used, each at two doses.**
- **The high dose of each compound was a known effect level.**
- **The low dose of VIN was expected to produce hypospadias and vaginal pouches that would be hard to detect in weanlings, but easier to detect in adults.**
- **The low dose of DBP was the LOAEL (lowest observable adverse effect level) for this compound.**
- **These compounds and the selected doses were identified by basic research protocols, and were used to test this hypothesis in rats.**

Vinclozolin (VIN)

Chemical Name:	3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione
CAS Number:	5-0471-44-8
Supplier:	Chem Services, Inc.
Manufacturer's Batch No.:	270-71B
Appearance:	colorless, crystalline solid
Molecular Formula:	$C_{12}H_9Cl_2NO_3$
Molecular Weight:	286.114
Structure:	
Common use:	systemic dicarboximide fungicide used on grapes, other fruit, vegetables, hops, ornamental plants and turf (Kelce et al., 1997).

Endocrine-Disrupting Properties of Vinclozolin

- van Ravenzwaay, 1992 (BASF study submitted to EPA)
  - Multigenerational studies in rats indicate that *in utero*/lactational exposure results in demasculinized male offspring.

- Gray et al., 1994:
  - 0, 100, or 200 mg/kg/day in corn oil, p.o., once daily, gd 14 - pnd 3 (rats)
  - **reduced anogenital** distance at birth
  - **nipple development** at 2 weeks
  - dose-related incidences and severities **cleft phallus with hypospadias, supra inguinal/ectopic scrota, vaginal pouch**, and **renal system malformations** at 1 year necropsy

- Gray et al., 1999:
  - 0, 3.125, 6.25, 12.5, 25, 50, and 100 mg/kg/day, p.o., gd 14 - pnd 3
  - **reduced anogenital distance, retained areolae**, and **permanent nipples** at $\geq$ 3.125 mg/kg/day
  - **reduced ventral prostate weight** at $\geq$ 6.25 mg/kg/day
  - **hypospadias** at 50 mg/kg/kday
  - **ectopic testes** at 100 mg/kg/day
  - The US EPA Reregistration Eligibility Decision document (2000) designated 6.0 mg/kg/day as the NOAEL, and 11.5 mg/kg/day as the adjusted LOAEL, based on these data.

Vinclozolin (continued)

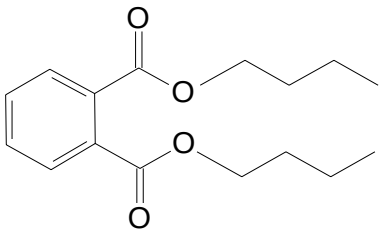
- Also, Hellwig et al., 2000
  - Wistar and Long-Evans rats, 0, 1, 3, 6, 12, or 200 mg/kg/day, p.o., gd 14 to pnd 3
  - **retained nipples/areolas** were present in both strains in preweanling males but persisted only in Long-Evans rats at 12 mg/kg/day. Long-Evans rats (but not Wistar) also exhibited a low incidence of **hypoplasia of accessory sex organs**.
  - male offspring from both strains exhibited **reduced anogenital distance, retained nipples/areolas, hypospadias, penile hypoplasia, vaginal pouch, hypoplasia** and **chronic inflammation of the epididymides, prostate, seminal vesicles**, and **coagulating glands, testicular tubule atrophy**, and **chronic inflammation of the urinary bladder**.

- *In vitro* studies indicate that the two vinclozolin metabolites bind to the androgen receptor (Kelce et al., 1994a) and, acting as antiandrogens, inhibit subsequent androgen receptor-dependent transcriptional activation (Wong et al., 1995). The adverse effects of vinclozolin are mediated by its metabolites (Kelce et al., 1994b). This mechanism has been confirmed *in vivo* (Kelce et al., 1997) with exposure to 200 mg/kg/day vinclozolin, resulting in alteration of the expression of androgen-dependent genes.

- The adult rat is also responsive to exposure to vinclozolin, but reproductive tract malformations are, as expected, not produced (Anderson et al., 1995).

- In light of the results of Gray et al., the doses for this study were 50 and 100 mg/kg/day

Di-butylphthalate (DBP)

CAS Number:	84-74-2
Supplier:	Sigma-Aldrich, Inc.
Manufacturer's Batch No.:	080K1023
Appearance:	clear, colorless liquid
Molecular Formula:	$C_{16}H_{22}O_4$
Molecular Weight:	278.35
Chemical Structure:	
Common use:	a coalescing aid in latex adhesives, as a plasticizer in cellulose plastics, and as a solvent in dyes.

Dibutylphthalate (DBP) *(continued)*

- Exposure during gestation results in developmental toxicity, and DBP crosses the placenta in rats (Saillenfait et al., 1998; Ema et al., 1993, 1994, 1995a, 1998). The intestinal metabolite, mono-n-butyl phthalate, also causes developmental toxicity in rats; this is most likely the proximate toxicant (Ema et al., 1995b).

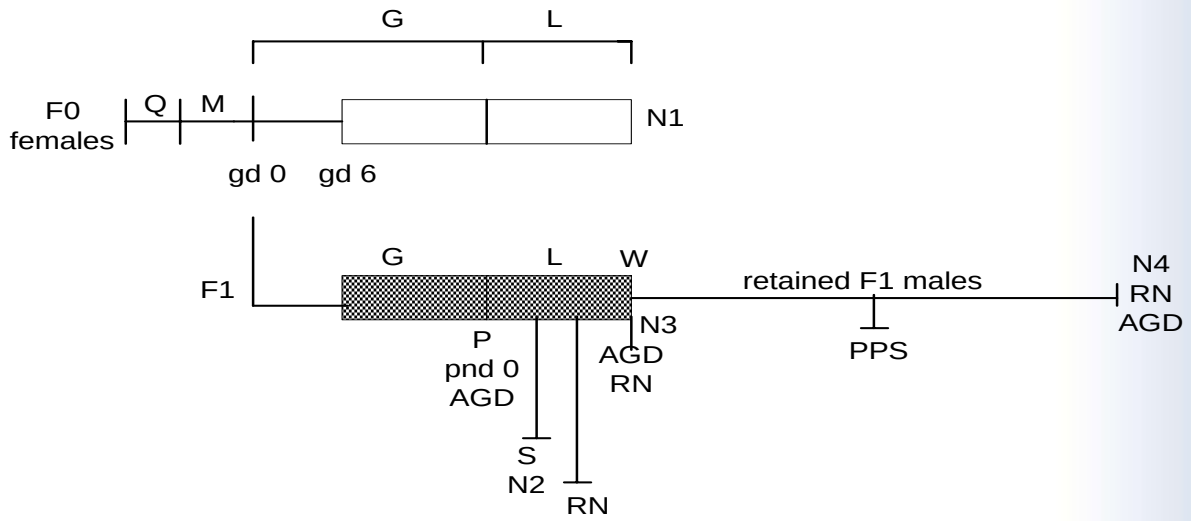
- Although DBP acts as an antiandrogen, it does not bind to the androgen receptor (Foster et al., 2000).

- DBP apparently acts by inhibiting fetal testicular testosterone biosynthesis *in vitro* and *in vivo* (Mylchreest et al., 1999). In adult rats, it also causes testicular toxicity but, as expected, no malformations (Cater et al., 1977). Daily oral (gavage) administration of DBP to dams, during gestation and lactation of 100 mg/kg/day through 750 mg/kg/day, results in dose-related reproductive malformations in male offspring, with approximately 75% of the male offspring affected at 750 mg/kg/day. The male malformations include shortened anogenital distance, small flaccid testes, agenesis of portions (caput, corpus, cauda) of or the entire epididymis, delayed puberty, retained nipples and areolae, etc. (Gray et al., 1998; 2000).

- An oral dose of 50 mg/kg/day has been defined as the NOAEL by Mylchreest et al. (1998a,b, 1999).

- Therefore, for this study, DBP in corn oil will be administered by oral gavage once daily on gd 6 through pnd 21 at 0 (vehicle control), 100 mg/kg/day (the LOAEL; lowest observed adverse effect level), and at 500 mg/kg/day (an obvious effect level).

One-Generation Extension Study Design



Key:

Direct dosing of F0 parental females, gd 6 - pnd 20

Possible indirect exposure of F1 offspring *in utero* and during lactation from transplacental and/or translactional transfer

No dosing of retained F1 males from weaning on pnd 21 to scheduled necropsy on pnd 95 ± 5

Q = quarantine (one week)
M = mating (one week)
G = gestation (~three weeks)
gd = gestational day
W = wean on pnd 21
S = standardize litters to 10 (with maximum number of males) on pnd 4
RN = examination of males for retained nipples
N1 = necropsy of F0 parental females at weaning of F1 litters
N2 = necropsy culled females to confirm sex
N3 = necropsy any remaining F1 females (and confirm sex), and necropsy three F1 males per litter at weaning on pnd 21
N4 = necropsy of retained F1 males at pnd 95 ± 5

L = lactation (three weeks)
pnd = postnatal day
P = parturition (date of birth, pnd 0)
AGD = anogenital distance
PPS = acquisition of preputial separation

RTI INTERNATIONAL

One-Generation Extension Study Target Doses

Group No.	No. F0 Dams Dosed	No. Days Exposure	Dosing Period (gd-pnd)	Dose (mg/kg/day)	Dosing Concentration (mg/kg/day)	Dose Volume (mL/kg)
1	25	36-38	6-21	0	0	5
2	25	36-38	6-21	50 VIN	10 VIN	5
3	25	36-38	6-21	100 VIN	20 VIN	5
4	25	36-38	6-21	100 DBP	20 DBP	5
5	25	36-38	6-21	500 DBP	100 DBP	5

Summary of F0 Maternal Data

- Mean periodic maternal body weights and weight gains
- Feed consumption (expressed as g/animal/day and g/kg body weight/day) during gestation and lactation
- Survival indices
- Gestational length
- Mean litter size
- Mean number of live and dead offspring
- Prenatal (postimplantation) loss (%) = $\frac{\text{No. implantation scars} - \text{No. live pups at birth}}{\text{No. implantation scars}} \times 100$
- Number and percent of mothers showing treatment-related behavioral abnormalities in nesting and nursing
- Gestational index (%) = $\frac{\text{No. pregnant females with live litters}}{\text{No. pregnant females}} \times 100$
- Gross necropsy
- Number of uterine nidation scars at necropsy

Summary of F1 Litter Lactational Data

- Total litter size
- Number and percent of stillborn
- Number and percent of live births
- Anogenital distance and body weight on pnd 0 and 21
- Periodic viability counts
- Periodic body weights by sex per litter from birth to weaning (taken on pnd 0, 4, 7, 14, and 21 by individual pup)
- Sex ratio (% males per litter)
- Presence of retained nipples and/or areolae in F1 offspring males on pnd 11-13, at weaning (pnd 21), and at adult necropsy (pnd 95 $\pm$ 5)

Summary of F1 Litter Lactational Data (cont'd)

Live Birth/Survival Indices:

Live birth index = $\frac{\text{No. live pups at birth}}{\text{Total no. pups at birth}} \times 100$

4-day survival index = $\frac{\text{No. pups surviving 4 days (precull)}}{\text{Total no. live pups at birth}} \times 100$

7-day survival index = $\frac{\text{No. pups surviving 7 days}}{\text{Total no. live pups at 4 days (postcull)}} \times 100$

14-day survival index = $\frac{\text{No. pups surviving 14 days}}{\text{Total no. live pups at 7 days}} \times 100$

21-day survival index = $\frac{\text{No. pups surviving 21 days}}{\text{Total no. live pups at 14 days}} \times 100$

Lactation index = $\frac{\text{No. pups surviving 21 days}}{\text{Total no. live pups at 4 days (postcull)}} \times 100$

Summary of Data From Retained Male F1 Offspring

- Mean periodic body weights and weight gains
- Age and body weight at acquisition of preputial separation
- Organ weights
- Reproductive system external and/or gross abnormalities
- Presence of areolae and/or nipples at adult necropsy
- Anogenital distance at adult necropsy

External and Internal Examination of F1 Males at Necropsy (pnd 21 or 95)

Each male selected for pnd 21 necropsy or pnd 95 adult necropsy was examined:

Externally

- Nipples and areolae were counted and position recorded
- hypospadias, epispadias, and cleft phallus
- AGD was measured
- Undescended testes
- Preputial separation
- Soiled perineum
- Vaginal pouch

Internally

- Location of each testis (scrotal, abdominal attached to abdominal wall)
- Gubernacular cords, present or absent and length in mm
- Presence of cranial suspensory ligaments and length in mm
- Testes which were small, absent, fluid filled, enlarged, appeared infected, or other
- Epididymides which were small, absent, or infected (including region of effects)
- Ventral prostate which was small, absent, or infected
- Dorsolateral prostate which was small, absent, or infected
- Seminal vesicles which were small, absent, infected, or one side larger than the other
- Coagulating glands which were small, absent, infected, one side larger than the other, or detached from seminal vesicles
- Vaginal pouch

External and Internal Examination of F1 Males at Necropsy (pnd 21 or 95) (cont'd)

- In addition the urinary system was evaluated as follows:
  - Kidneys with hydronephrosis or calcium deposits
  - Hydroureter(s)
  - Urinary bladder stones or blood in urinary bladder

- The following organs were weighed:
  - Each testis individually
  - Each corpus plus caput epididymides
  - Each cauda epididymides
  - Entire seminal vesicle, plus coagulating glands with fluid as a unit, if possible
  - The prostate ventral and dorsolateral lobes separately
  - Paired adrenals
  - Liver
  - Levator ani plus bulbocavernosus (LABC) muscle complex
  - Cowper's (bulbourethral) glands as a pair
  - Glans penis (only if preputial separation has occurred)

Pnd 21 Necropsy

	Guideline Two-Gen Study	Extended One-Gen
Weights:		
Body	X	X
Brain	X	
Spleen	X	
Thymus	X	
Liver		X
Adrenal		X
Testis		X
Corpus/Caput Epididymis		X
Cauda Epididymis		X
Seminal Vesicles/Coagulating Gland		X
Prostate		X
LABC		X
Cowper's Glands		X

Pnd 21 Necropsy (cont'd)

	Guideline Two-Gen Study	Extended One-Gen
AGD		X
Areolae		X
Nipples		X
Hypospadias		X
Epispadias		X
Cleft Phallus		X
Soiled Inguinal Region		X
Gubernacular Cord [a]		X
Cranial Suspensory Ligaments [a]		X
Histopathology of Reproductive Anomalies observed macroscopically	X	

[a] Present/Absent; Length

Pnd 95 Necropsy

	Guideline Two-Gen Study (F1 Parental Males)	Extended One-Gen
Weights:		
Body	X	X
Brain	X	
Liver	X	X
Kidney	X	
Adrenal	X	X
Spleen	X	
Testis	X	X
Total Epididymis	X	
Corpus/Caput Epididymis		X
Cauda Epididymis	X	X
Seminal Vesicles/Coagulating Gland	X	X
Prostate	X	X
Ventral		X
Dorsal		X
LABC		X
Cowper's Glands		X
		X

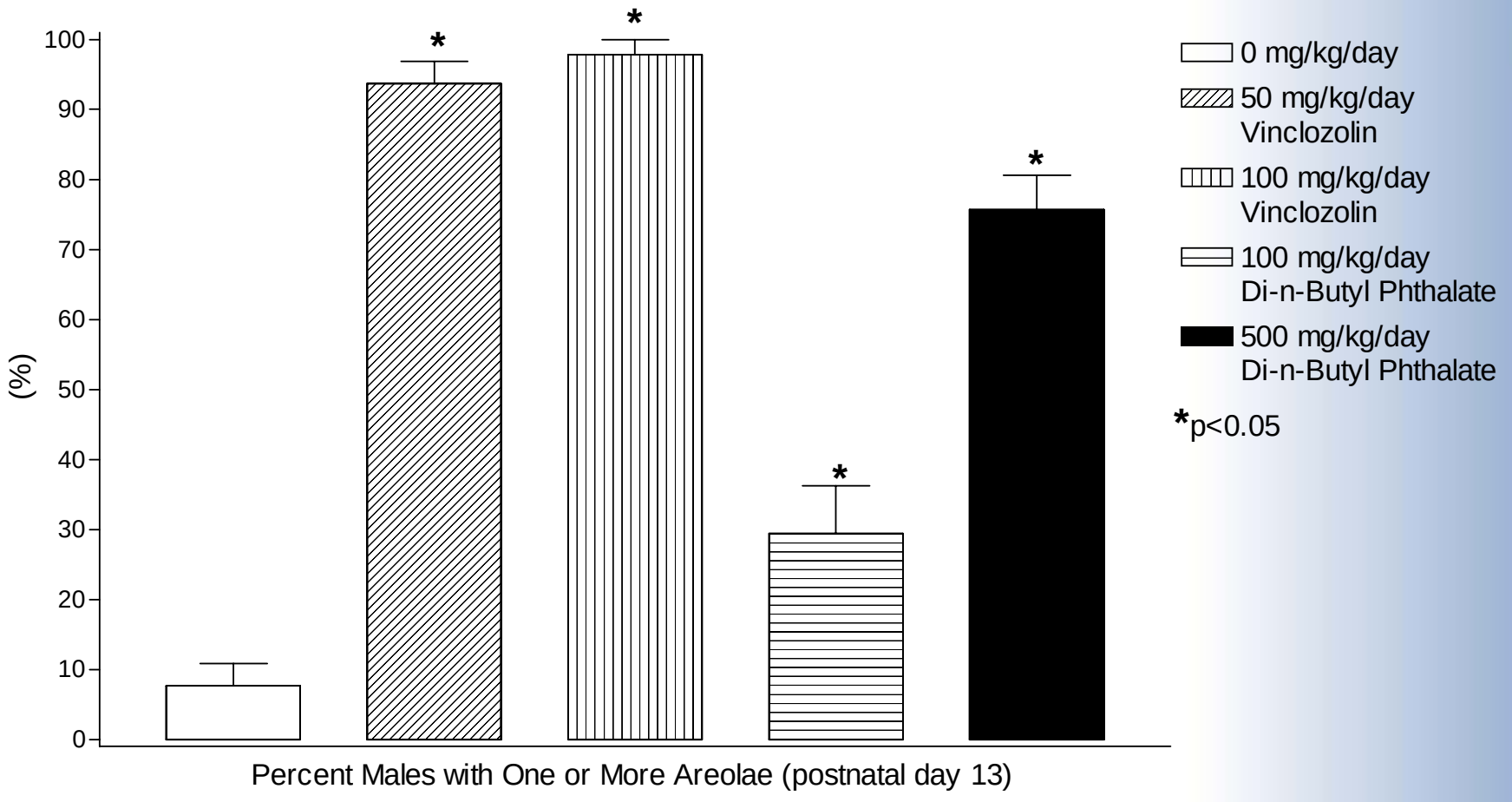
Pnd 95 Necropsy

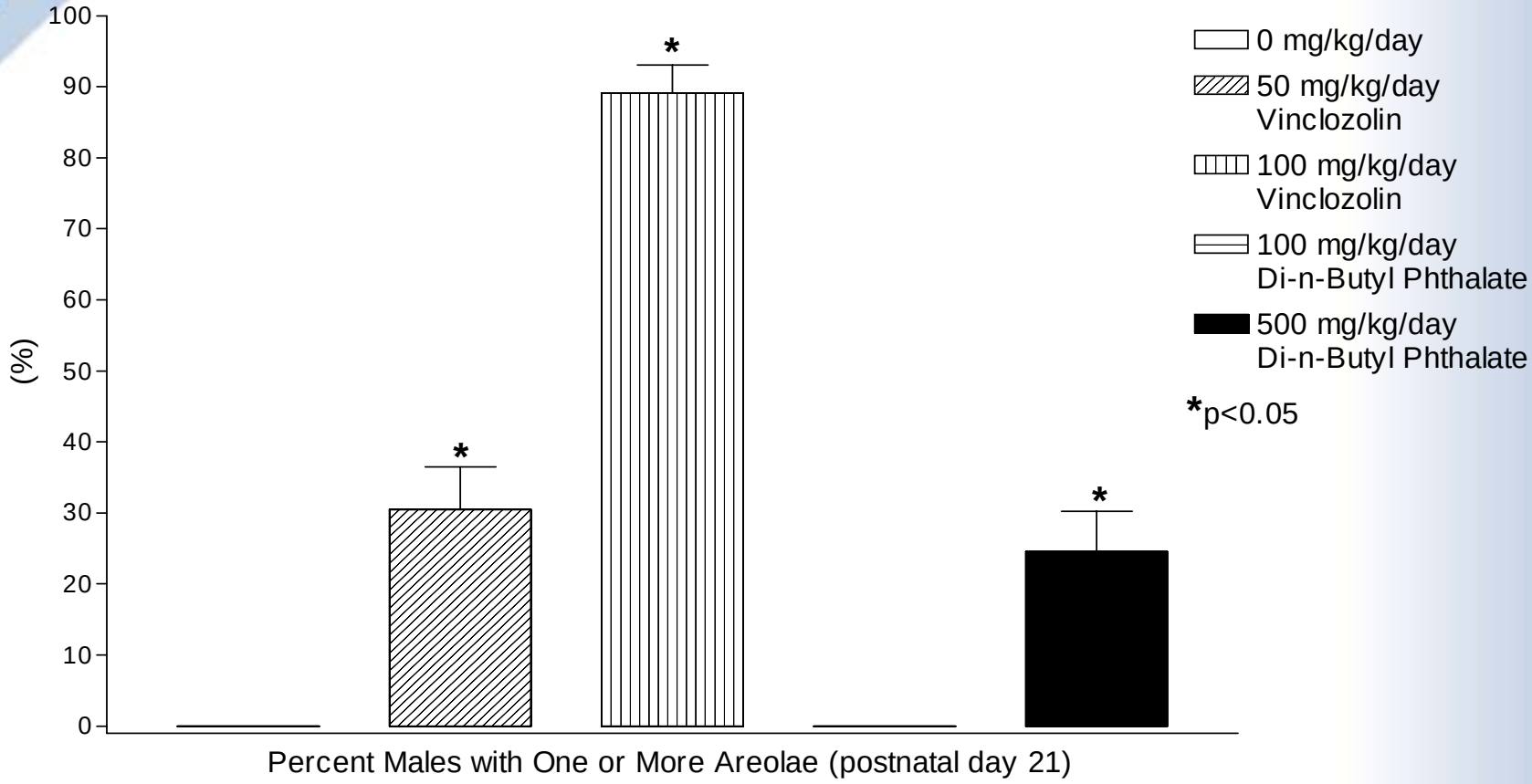
	Guideline Two-Gen Study	Extended One-Gen
AGD		X
Areolae		X
Nipples		X
Hypospadias		X
Epispadias		X
Cleft Phallus		X
Soiled Inguinal Region		X
Gubernacular Cord [a]		X
Cranial Suspensory Ligaments [a]		X
Histopathology	Weighed Tissues	

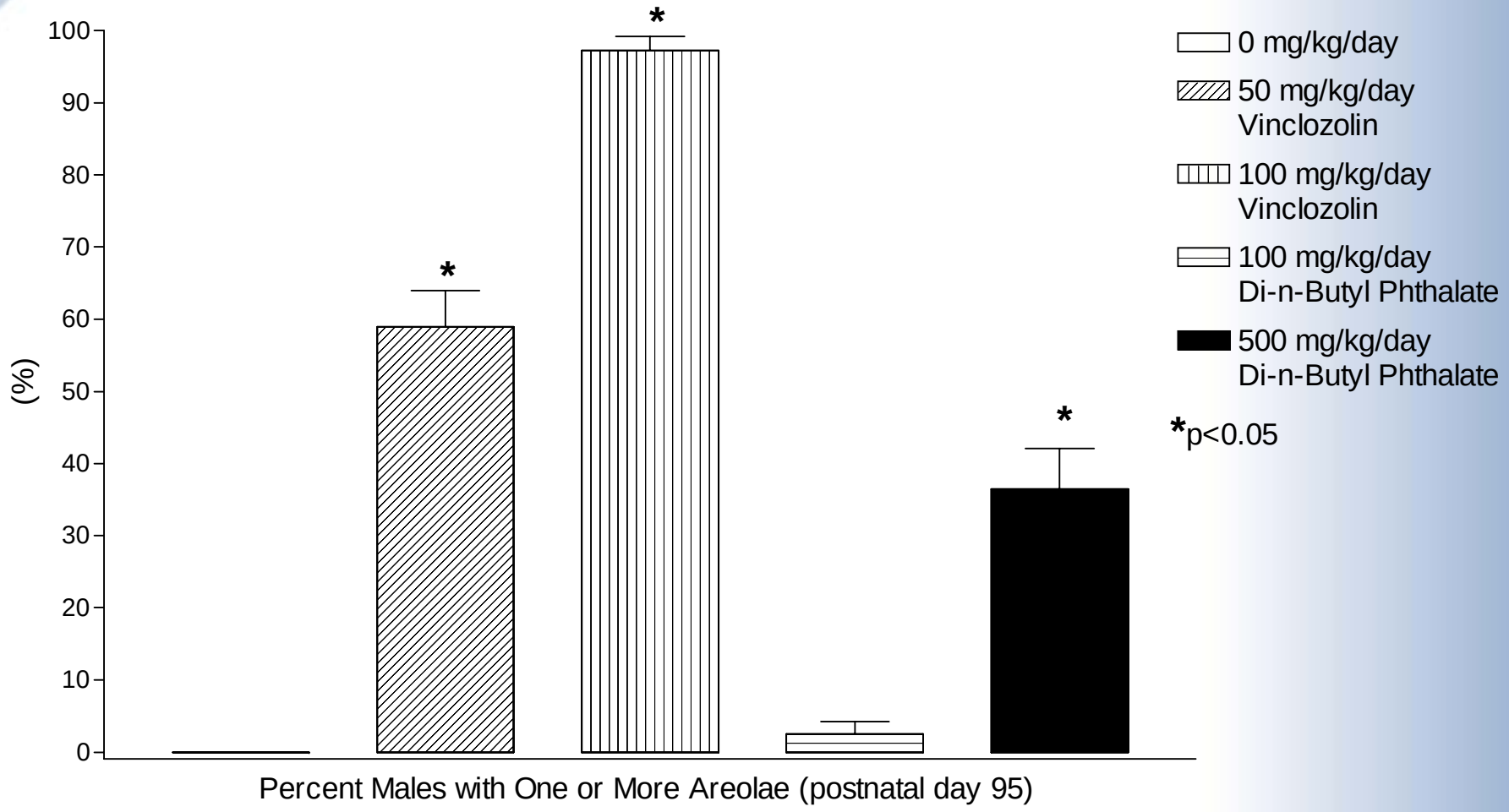
[a] Present/Absent; Length

Can this study design and the performing laboratory detect both doses of both test compounds (VIN and DBP) as effect levels?

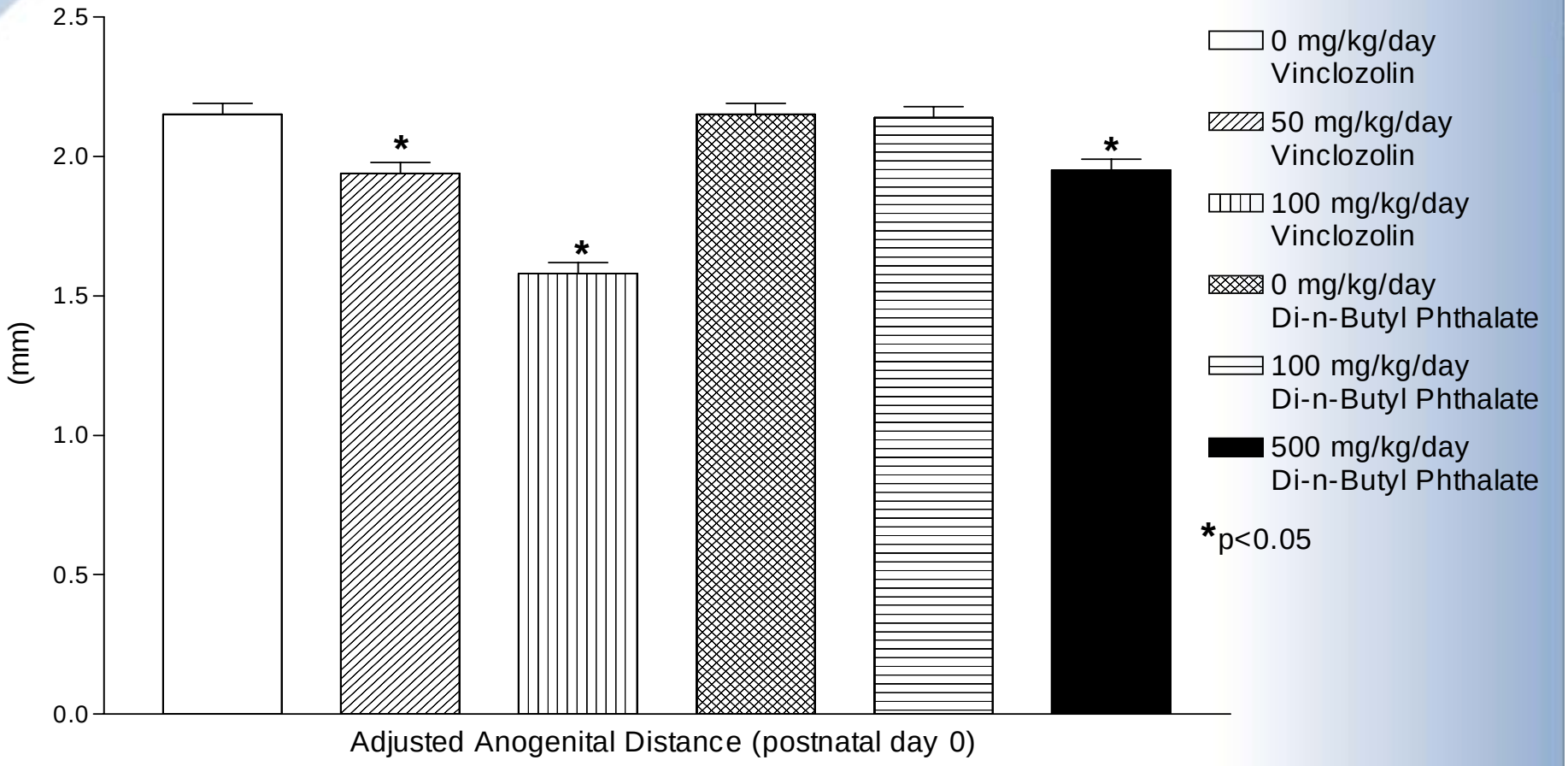
Both doses of both test chemicals were detected as effect levels in preweanling males by pnd 13, based on retention of areolae.

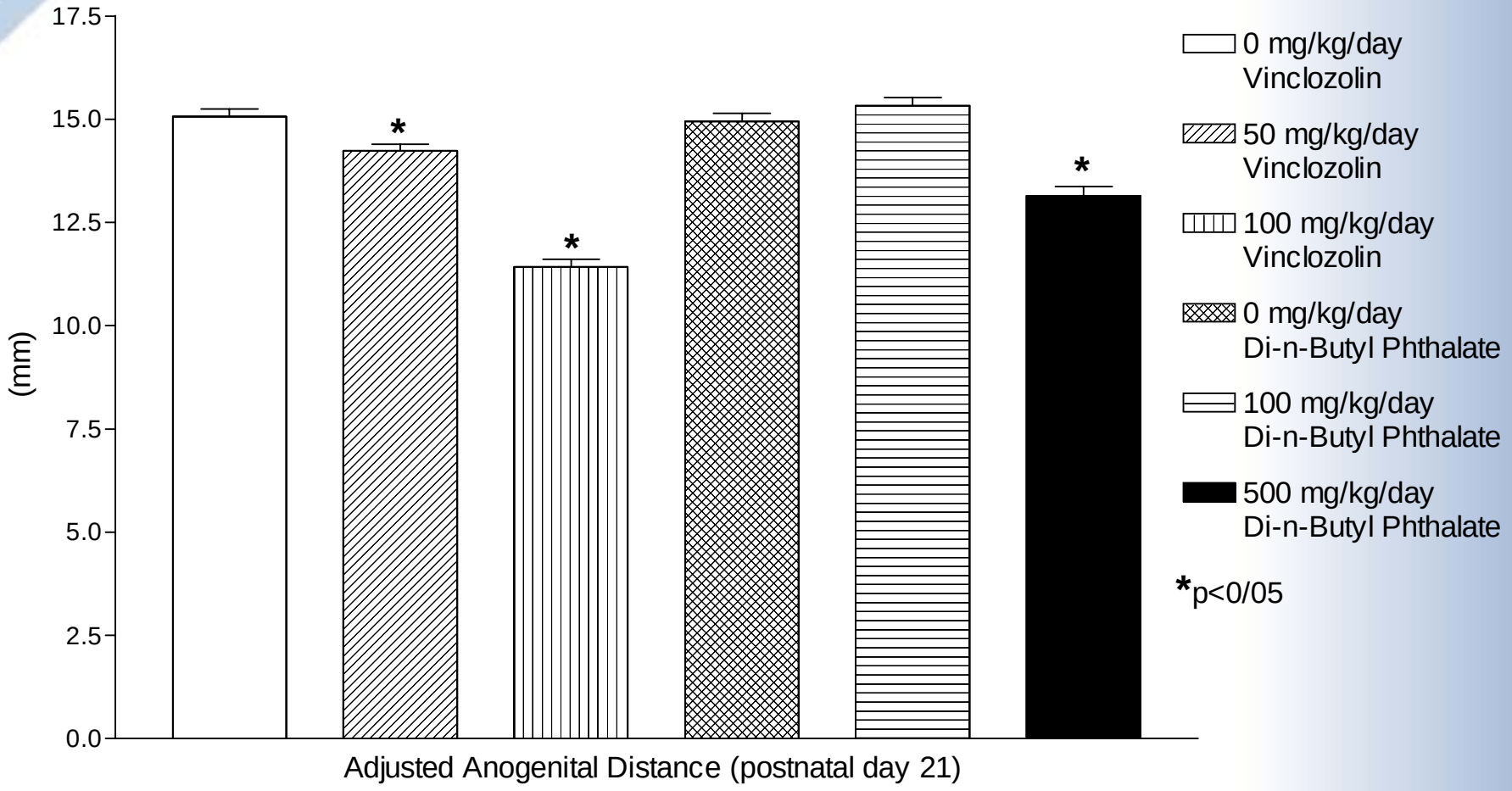

100
90
80
70
60
50
40
30
20
10
0
(%)
*
*
*
*
0 mg/kg/day
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05
Percent Males with One or More Areolae (postnatal day 13)

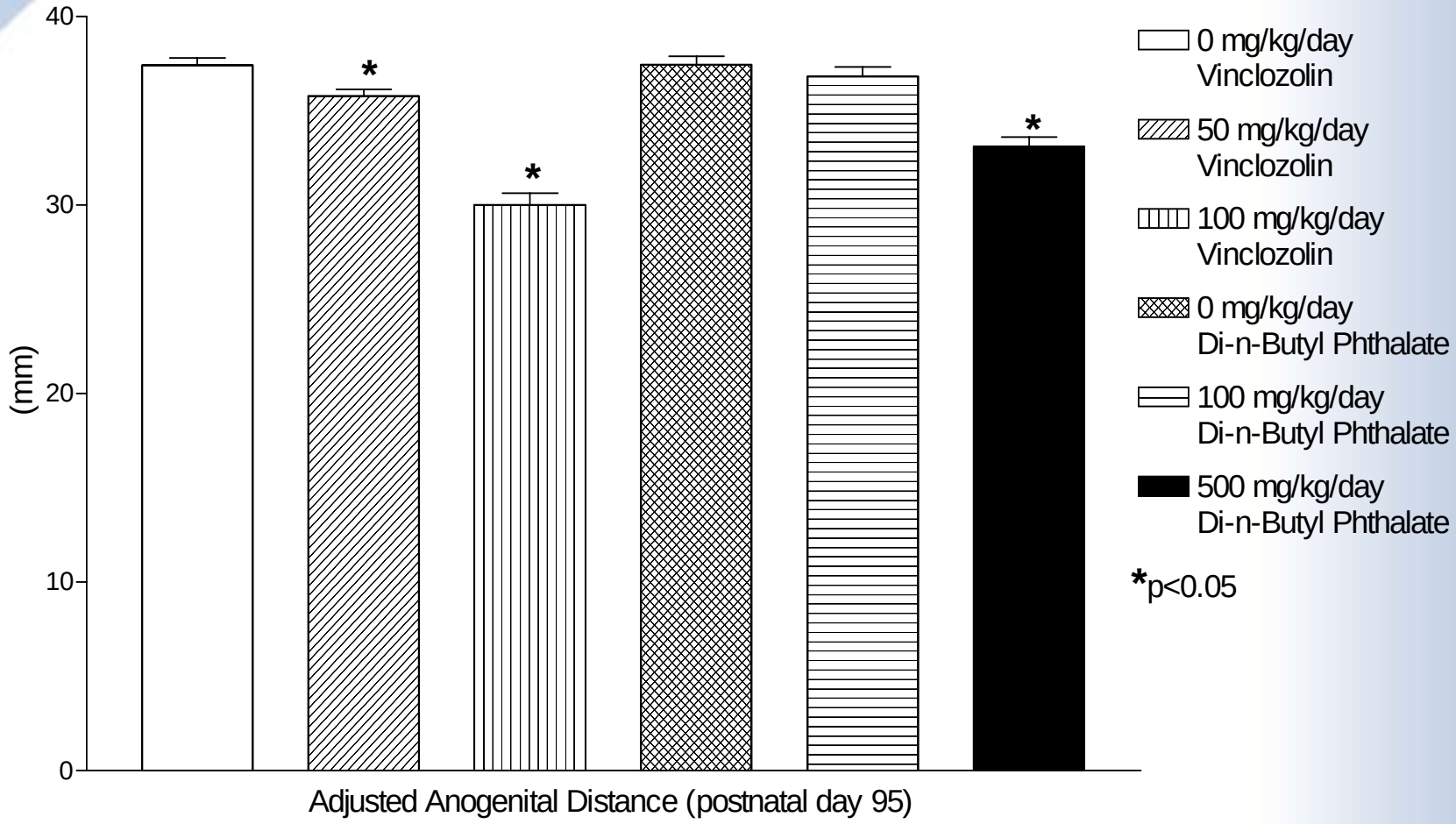

100
90
80
70
60
50
40
30
20
10
0
(%)
*
*
*
0 mg/kg/day
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05
Percent Males with One or More Areolae (postnatal day 21)


100
90
80
70
60
50
40
30
20
10
0
(%)
*
*
*
0 mg/kg/day
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05
Percent Males with One or More Areolae (postnatal day 95)

Both doses of VIN and the high dose of DBP were detected as effect levels from data on AGD at birth. This effect persisted throughout the study to pnd 95.

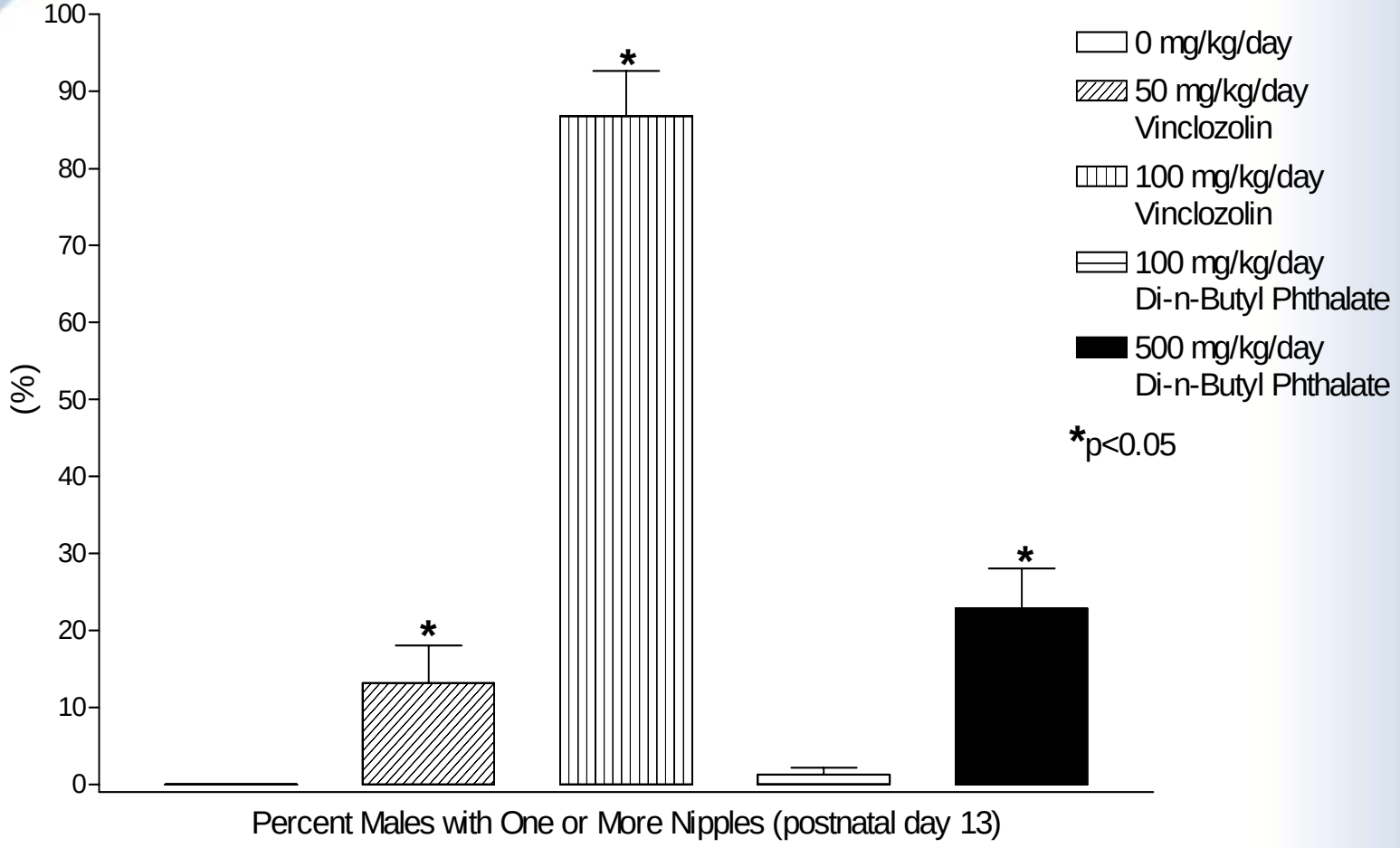

2.5
2.0
1.5
1.0
0.5
0.0
(mm)
*
*
*
Adjusted Anogenital Distance (postnatal day 0)
0 mg/kg/day Vinclozolin
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
0 mg/kg/day Di-n-Butyl Phthalate
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05


17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
(mm)
*
*
*
0 mg/kg/day Vinclozolin
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
0 mg/kg/day Di-n-Butyl Phthalate
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0/05
Adjusted Anogenital Distance (postnatal day 21)

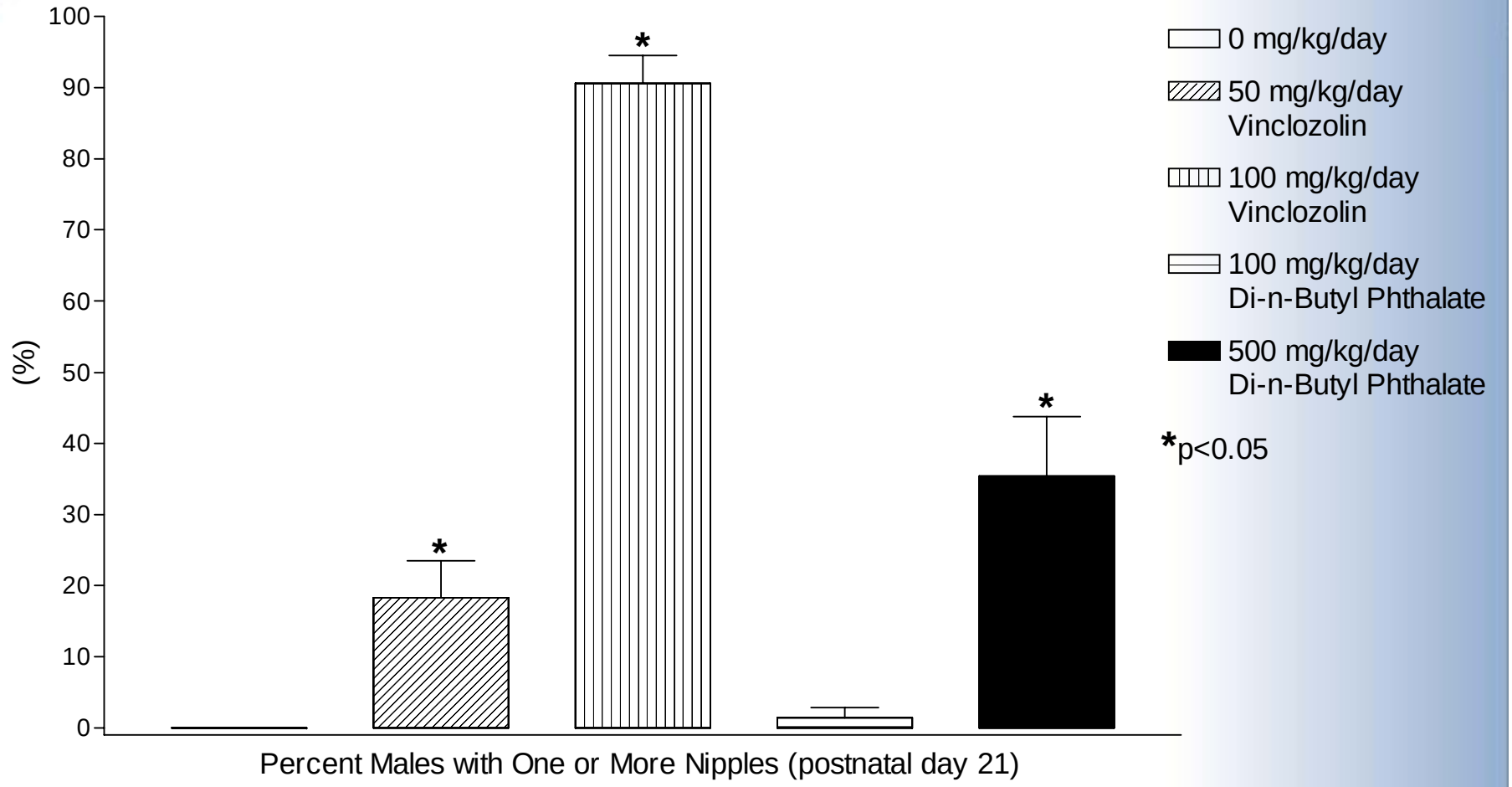

40
30
20
10
0
(mm)
*
*
*
Adjusted Anogenital Distance (postnatal day 95)
0 mg/kg/day Vinclozolin
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
0 mg/kg/day Di-n-Butyl Phthalate
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05

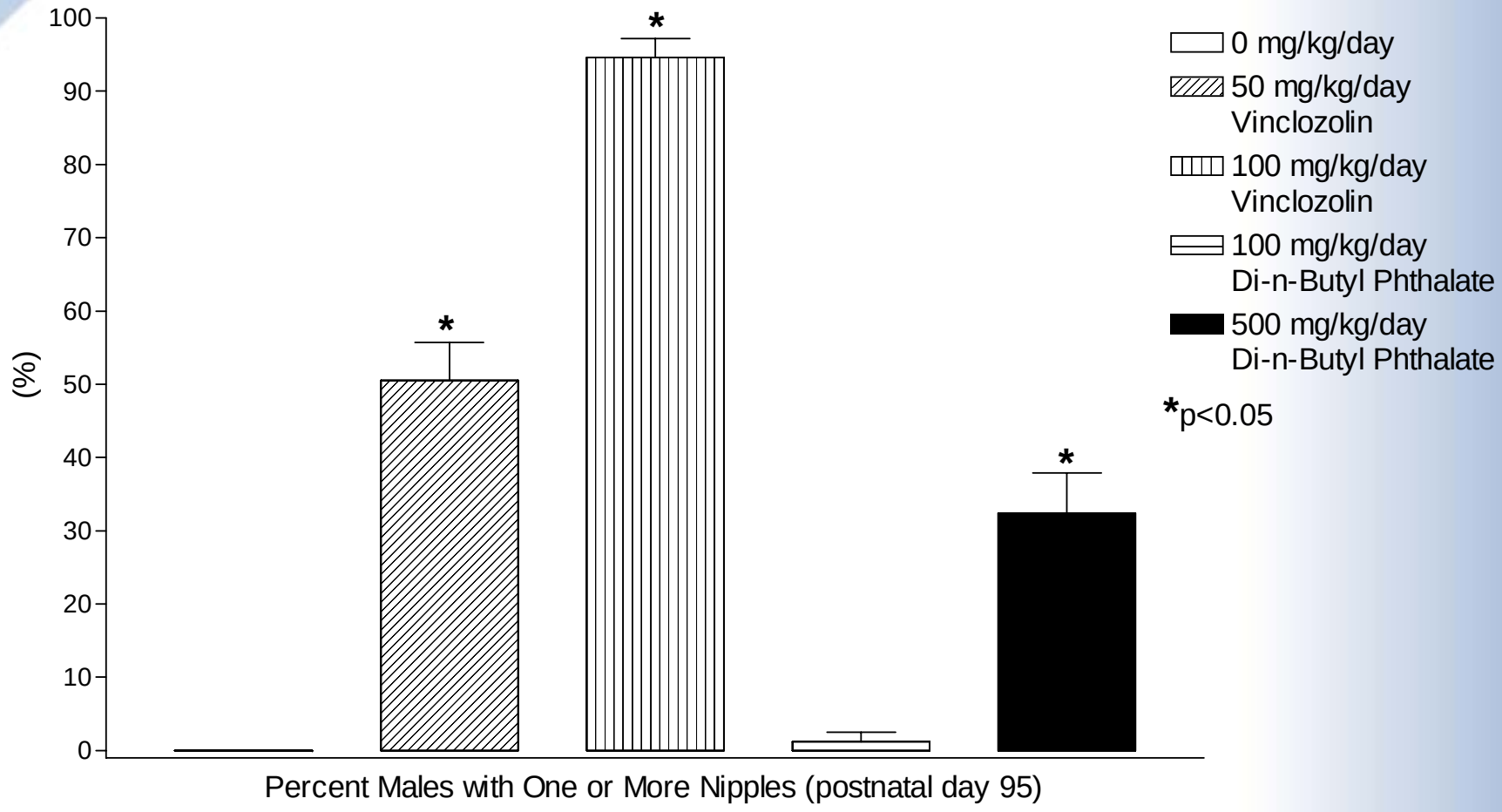
The incidence of nipples on pnd 11-13 males was significantly increased for both doses of VIN and for the high dose (but not the low dose) of DBP.

Since retained nipples are not observed in control males, and retained areolae are (at 0-3.5% incidence in the performing laboratory), the anticipation was that retained nipples would be a more sensitive indicator of anti-androgenic activity than retained areolae, but retained areolae are a more sensitive indicator.



RTI INTERNATIONAL


100
90
80
70
60
50
40
30
20
10
0
(%)
*
*
*
*
0 mg/kg/day
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05
Percent Males with One or More Nipples (postnatal day 21)


100
90
80
70
60
50
40
30
20
10
0
(%)
*
*
*
0 mg/kg/day
50 mg/kg/day Vinclozolin
100 mg/kg/day Vinclozolin
100 mg/kg/day Di-n-Butyl Phthalate
500 mg/kg/day Di-n-Butyl Phthalate
*p<0.05
Percent Males with One or More Nipples (postnatal day 95)

Were male reproductive system malformations detected at the pnd 21 necropsy for both test chemicals at both doses?

F1 Male Offspring Reproductive System Malformations at the Pnd 21 Necropsy

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. pups	74	82	65	71	65
Cowper's gland missing	**0 (0.0)**	**9 (11.0)**	**47 (72.3)**	**1 (1.4)**	**7 (10.8)**
Epididymis(mides) missing, reduced in size:	**0 (0.0)**	**0 (0.0)**	**2 (3.1)**	**0 (0.0)**	**21 (32.3)**
Epispadias	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hypospadias	0 (0.0)	**8.0 (9.7)**	**52 (80.0)**	0 (0.0)	2 (3.1)
Males with ≥ 1 gubernacular cord	**74 (100.0)**	**82 (100.0)**	**65 (100.0)**	**69 (97.2)**	**59 (90.8)**
Males with ≥ 1 cranial suspensory ligament	0 (0.0)	1 (1.3)	0 (0.0)	2.2 (3.1)	2.2 (3.4)
Levator ani bulbo-cavernosus complex:					
Missing	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Spongy	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Penis reduced in size	0 (0.0)	0 (0.0)	3 (4.6)	0 (0.0)	0 (0.0)
Phallus, cleft	0 (0.0)	4 (4.9)	25 (38.5)	0 (0.0)	2 (3.1)
Prostate missing:					
Dorsal	0	0	21 (32.3)	0	1 (1.5)
Ventral	0	0	4 (6.2)	0	1 (1.5)
V left lobe	0	0	1 (1.5)	0	1 (1.5)
Total	**0 (0.0)**	**0 (0.0)**	**26 (40.0)**	**0**	**3 (4.6)**
Seminal vesicles, missing/misshapen	**0 (0.0)**	**0 (0.0)**	**7 (10.8)**	**0**	**7 (10.8)**
Testes undescended	0 (0.0)	0 (0.0)	3 (4.6)	0	4 (6.1)
Hydronephrosis: [b]	**3 (4.0)**	**5 (6.1)**	**0 (0.0)**	**3 (4.2)**	**6 (9.2)**

[a] Number (and %) with the indicated finding. A male may be counted more than once if he exhibited more than one malformation.

[b] The incidence of hydronephrosis, a common finding in male CD® (Sprague-Dawley) rats is provided for internal quality control. There was no chemical- or dose-related incidence.

In utero/lactational exposure to both doses of both test chemicals resulted in male reproductive system malformations in a dose-related incidence and severity.

The male reproductive malformations at the low dose of DBP were biologically significant (never observed in controls) but clearly not statistically significant. They included missing Cowper's glands and presence of cranial suspensory ligaments (normally observed only in females).

Were male reproductive system malformations detected at pnd 95

for both test chemicals at both doses?

F1 Male Offspring Reproductive System Malformations at the Pnd 95 Necropsy[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males	82	95	74	81	74
Cowper's glands:					
Missing	0 (0.0)	6 (6.32)	56 (75.7)	0 (0.0)	5 (6.8)
Reduced in size	0 (0.0)	1 (1.0)	8 (10.8)	0 (0.0)	3 (4.0)
Epididymis missing	0 (0.0)	0 (0.0)	4 (5.4)	0 (0.0)	33 (44.6)
Reduced in size	1 (1.2)	0 (0.0)	12 (16.2)	0 (0.0)	52 (70.3)
Epispadias	0 (0.0)	4 (4.3)	11 (14.9)	0 (0.0)	0 (0.0)
Glans penis not completely detached [b]	0 (0.0)	3 (3.2)	20 (27.0)	0 (0.0)	7 (9.5)
Hypospadias	0 (0.0)	15 (15.8)	73 (98.6)	0 (0.0)	12 (16.2)
LABC: [b]					
Missing	0 (0.0)	0 (0.0)	2 (2.7)	0 (0.0)	0 (0.0)
Reduced in size	0 (0.0)	2 (2.1)	38 (51.4)	0 (0.0)	4 (5.4)
Malformed	0 (0.0)	1 (1.0)	5 (6.7)	0 (0.0)	0 (0.0)
Males with $\geq$ 1 gubernacular cord	5.5 (6.2)	11 (11.6)	13 (17.6)	1.0 (1.2)	6 (8.1)
Males with $\geq$ 1 cranial suspensory ligament	0 (0.0)	0 (0.0)	1.0 (1.4)	0 (0.0)	6.2 (8.4)
Phallus, cleft	1 (1.20)	41 (43.2)	74 (100.0)	2.0 (2.5)	26 (35.1)
Prepuce partially or fully detached	81 (98.8)	94 (99.0)	74 (100.0)	81 (100.0)	74 (100.0)
Preputial glands, pus filled [b]	0 (0.0)	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)
Prostate, dorsal:					
Missing	0 (0.0)	0 (0.0)	17 (23.0)	0 (0.0)	3 (4.0)
Reduced in size	0 (0.0)	2 (2.4)	20 (27.0)	2 (2.5)	7 (9.5)
Abnormal/infected	0 (0.0)	2 (2.4)	3 (4.0)	0 (0.0)	1 (1.4)

(Continued)

F1 Male Offspring Reproductive System Malformations at the Pnd 95 Necropsy[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
Prostate, ventral:					
Missing	0 (0.0)	0 (0.0)	12 (16.2)	0 (0.0)	3 (4.0)
Reduced in size	0 (0.0)	4 (4.9)	43 (58.1)	1 (1.2)	4 (5.4)
Abnormal/infected	1 (1.2)	3 (3.1)	5 (6.7)	1 (1.2)	6 (8.1)
Seminal vesicles:					
Missing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (10.8)
Misshapen/infected	0 (0.0)	0 (0.0)	6 (8.1)	0 (0.0)	5 (6.8)
Reduced in size	0 (0.0)	5 (6.1)	58 (78.4)	0 (0.0)	27 (36.5)
Testes					
Undescended	0 (0.0)	1 (1.0)	15 (20.3)	0 (0.0)	10 (13.5)
wall[c] sc in abdom.	0 (0.0)	0 (0.0)	8 (10.8)	0 (0.0)	2 (2.7)
Reduced in size	1 (1.2)	1 (1.0)	17 (23.0)	0 (0.0)	45 (60.8)
Flaccid/soft	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	32 (43.2)
Enlarged	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.2)	2 (2.7)
Abnormal/infected	0 (0.0)	0 (0.0)	3 (4.0)	0 (0.0)	1 (1.4)
Urinary bladder: [b]					
Adhered to prostate	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	0 (0.0)
Calculi present	1 (1.2)	0 (0.0)	3 (4.0)	0 (0.0)	1 (1.4)
Vaginal pouch [b]	0 (0.0)	2 (2.4)	43 (58.1)	0 (0.0)	1 (1.4)

[a] Data are presented as number (%) with the indicated finding; data from summary tables 20 and 21. On this table, a male may be counted more than once if he exhibited more than one malformation.
[b] Findings not reported at the pnd 21 necropsy.
[c] Undescended testes imbedded subcutaneously (sc) in the abdominal wall.

In utero/lactational exposure to both test chemicals at both doses resulted in male reproductive system malformations in a dose- and chemical-related incidence and severity on pnd 95.

For the low dose DBP, findings included cleft phallus, dorsal and ventral prostate lobes reduced in size, and enlarged testes.

Admittedly, these were observed at a low incidence at this dose, but they were biologically significant although not likely statistically significant.

Did the F1 males that died or were sacrificed moribund also exhibit male reproductive malformations?

There were two F1 males each at the VIN high dose and the DBP high dose that died or were sacrificed moribund. They did exhibit the same male reproductive system malformations as those observed in the adult males at scheduled necropsy.

Were the incidences of findings present at both pnd 21 and 95 necropsies different?

- 19 effects were observed on pnd 95 that were not observed on pnd 21

Were they greater on pnd 95?

- in all but one finding (Cowper's glands missing/reduced in size) the incidence was higher on pnd 95 than on pnd 21

Would they have been detected on pnd 95 with only one male evaluated per litter?

Comparison of the Incidence of Male Reproductive System Malformations on pnd 21 Versus pnd 95

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males, pnd 21	74	82	65	71	65
No. males, pnd 95	82	95	74	81	74
Cowper's gland missing/ reduced in size:					
Pnd 21	0 (0.0)[a]	9 (11.0)	47 (72.3)	1 (1.4)	7 (10.8)
Pnd 95	1 (1.2)	7 (7.4)	63 (85.1)	0 (0.0)	8 (10.8)
Epididymides missing					
Pnd 21	0 (0.0)	0 (0.0)	2 (3.1)	0 (0.0)	14 (21.5)
Pnd 95	0 (0.0)	0 (0.0)	4 (5.4)	0 (0.0)	33 (44.6)
Epididymides reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (6.2)
Pnd 95	1 (1.2)	0 (0.0)	19 (25.7)	0 (0.0)	52 (71.6)
Epispadias:					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	4 (4.3)	11 (14.9)	0 (0.0)	0 (0.0)
Hypospadias:					
Pnd 21	0 (0.0)	8.0 (9.7)	52 (80.0)	0 (0.0)	2 (3.1)
Pnd 95	0 (0.0)	15 (15.8)	73 (98.6)	0 (0.0)	12 (16.2)
LABC missing/reduced in size:					
Pnd 21	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	2 (2.1)	40 (54.0)	0 (0.0)	4 (5.4)
Parameter	0	50	100	100	500
Phallus, cleft:					
Pnd 21	0 (0.0)	4 (4.9)	25 (38.5)	0 (0.0)	2 (3.1)
Pnd 95	1 (1.2)	41 (43.15)	74 (100.0)	2.0 (2.47)	26 (35.14)
Prostate dorsal lobe missing					
Pnd 21	0 (0.0)	0 (0.0)	21 (32.3)	0 (0.0)	1 (1.5)
Pnd 95	0 (0.0)	0 (0.0)	17 (23.0)	0 (0.0)	3 (4.0)
Prostate dorsal lobe reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	3 (3.2)	20 (27.0)	2 (2.5)	8 (10.8)
Prostate ventral lobe missing					
Pnd 21	0 (0.0)	0 (0.0)	5 (7.7)	0 (0.0)	2 (3.1)
Pnd 95	0 (0.0)	0 (0.0)	12 (16.2)	0 (0.0)	3 (4.0)

(Continued)

F1 Male Offspring Reproductive System Malformations at the Pnd 95 Necropsy[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
Prostate ventral lobe reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	1 (1.2)	7 (7.4)	45 (60.8)	2 (2.5)	8 (10.8)
Seminal vesicles missing/ reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	8 (12.3)	0 (0.0)	7 (10.8)
Pnd 95	0 (0.0)	5 (6.1)	63 (85.1)	0 (0.0)	39 (52.7)
Testes undescended					
Pnd 21	0 (0.0)	0 (0.0)	3 (4.6)	0 (0.0)	4 (6.1)
Pnd 95	0 (0.0)	1 (1.0)	15 (20.3)	0 (0.0)	10 (13.3)
Testes reduced in size					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	1 (1.2)	1 (1.0)	17 (23.0)	0 (0.0)	45 (60.8)
Testes flaccid/soft					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	32 (43.2)
Males with >1 gubernacular wt.[b]					
Pnd 21	74 (100.0)	82 (100.0)	65 (100.0)	69 (97.2)	59 (90.8)
Pnd 95	5.5 (6.2)	11 (11.6)	13 (17.6)	1.0 (1.2)	6 (8.1)
Males with >1 cranial suspensory ligament[b]					
Pnd 21	0 (0.0)	1 (1.32)	0 (0.0)	2.2 (3.1)	2.2 (3.4)
Pnd 95	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	6.2 (8.1)
Vaginal pouch					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	2 (2.4)	43 (58.1)	0 (0.0)	1 (1.4)

[a] Data presented as number (%) of males exhibiting the finding. Males may be counted more than once if they exhibited more than one finding.

[b] The incidences of these findings are taken from Summary Table 13 (and Text Table 3) for pnd 21, and from Summary Table 20 (and Text Table 4) for pnd 95.

Male malformations observed on pnd 95 but not on pnd 21 in either or both test chemicals or in both dose groups per chemical include the following:

- epididymides reduced in size/abnormal (not observed in either VIN groups on pnd 21)
- epispadias (not detected in any group on pnd 21)
- levator ani bulbocavernosus (LABC) complex, missing/reduced in size (not observed in the low dose of VIN or either dose of DBP on pnd 21)
- cleft phallus (not observed in the low dose of DBP on pnd 21)
- dorsal and/or ventral lobes of prostate reduced in size/abnormal (not detected in any group on pnd 21)
- seminal vesicles missing/reduced in size/abnormal (not observed in the low dose of VIN on pnd 21)
- undescended testes (not observed in the low dose of VIN on pnd 21)
- testes reduced in size or flaccid/soft (not detected in any group on pnd 21)
- vaginal pouch (not detected in any group on pnd 21)

Were there differences in the effects on weights of various male reproductive system organs between the two chemicals at the same time point?

VIN:
Seminal vesicle and coagulating gland ↓
prostate ↓
LABC ↓
Cowper's glands ↓

DBP:
testes ↓
epididymides ↓

Were there differences in the effects on weights of various male reproductive system organs within each chemical at the two different time points?

Comparison of Incidences of Male Reproductive System Malformations on Pnd 21 and Pnd 95

Parameter	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
	0	50	100	100	500
Pnd 21[a]					
No. males examined	74	82	65	71	65
No. litters examined	23	25	22	23	23
Pnd 95[b]					
No. males examined	82	95	74	81	74
No. litters examined	23	25	22	23	23
Incidence of total no. of malformations based on no. F1 males examined					
Pnd 21 [c,d]	0 (0.0)	21 (25.6)	169 (260.0)	1 (1.4)	43 (66.2)
Pnd 95 [c,e]	8 (9.8)[f]	95 (100.0)	532 (718.9)	11 (13.6)	237 (320.3)
Total number of F1 males with ≥1 malformations					
Pnd 21 [c,d,g]	0 (0.0)	9 (11.0)	54 (83.1)	1 (1.4)	29 (44.6)
Pnd 95 [c,e]	7 (8.5)[f]	47 (49.5)	74 (100.0)	7 (8.6)	68 (91.9)
Total no. of F1 litters with ≥ 1 male with ≥ 1 malformation					
Pnd 21 [c,d,g]	0 (0.0)	7 (28.0)	21 (95.5)	1 (4.4)	21 (91.3)
Pnd 95 [c,e]	7 (30.4)[f]	21 (84.0)	22 (100.0)	7 (30.4)	22 (95.7)
No. malformed males per affected litters					
Pnd 21 [c,d,g]	0.00	1.29	2.57	1.00	1.38
Pnd 95	1.00[f]	2.24	3.36	1.00	3.09

[a] The number of F1 male weanlings/litter examined in this protocol versus the numbers from the 1998 OPPTS testing guideline are approximately equivalent (3.22-3.54 vs. 3).

[b] The OPPTS testing guideline specifies one male/litter retained to adulthood. Therefore, this protocol provides at least three times the sensitivity to detect adult male malformations (3.22-3.80 vs. 1).

[c] Data presented as number (%).

[d] Pnd 21 data taken from Summary Table 14 and Individual Animal Tables A-15 and A-17.

[e] Pnd 95 data taken from Summary Table 21 and Individual Animal Tables A-26 and A-28.

[f] Predominantly minor effects (e.g., epididymis and testis reduced in size, prostate abnormal [hard and brown], etc.).

[g] Predominantly missing Cowper's gland(s).

Comparison of Male Reproductive System Organ Weights on pnd 21 versus pnd 95[a]

Parameter	Vehicle Control (mg/kg/day) 0	VIN (mg/kg/day) 50	VIN (mg/kg/day) 100	DBP (mg/kg/day) 100	DBP (mg/kg/day) 500
Testis					
Right Ab pnd 21	— (0.1323)	—	↓ ↓ (0.1198)	—	↓ ↓ ↓ (0.1017)
Right Ab pnd 95	— (1.7702)	—	↓ ↓ ↓ (1.5284)	—	↓ ↓ ↓ (1.2383)
Right Ajpnd 21	— (0.1295)	↑ (0.1334)	—	↓ (0.1221)	↓ ↓ ↓ (0.1105)
Right Ajpnd 95	— (1.7680)	—	↓ ↓ ↓ (1.5335)	—	↓ ↓ ↓ (1.2416)
Left Ab pnd 21	— (0.1305)	—	↓ ↓ (0.1186)	—	↓ ↓ ↓ (0.0983)
Left Ab pnd 95	— (1.7688)	—	↓ ↓ ↓ (1.4970)	—	↓ ↓ ↓ (1.3982)
Left Ajpnd 21	— (0.1281)	—	—	↓ ↓ (0.1189)	↓ ↓ ↓ (0.1078)
Left Ajpnd 95	— (1.7649)	—	↓ ↓ ↓ (1.5056)	—	↓ ↓ ↓ (1.4089)
Corpus and Caput Epididymis					
Right Ab pnd 21	— (0.0139)	↓ (0.0125)	↓ ↓ ↓ (0.0100)	↓ (0.0123)	↓ ↓ ↓ (0.0088)
Right Ab pnd 95	— (0.3623)	—	↓ ↓ ↓ (0.3267)	—	↓ ↓ ↓ (0.2405)
Right Ajpnd 21	— (0.0137)	↓ (0.0123)	↓ ↓ ↓ (0.0105)	↓ (0.0121)	↓ ↓ ↓ (0.0094)
Right Ajpnd 95	— (0.3617)	—	↓ ↓ ↓ (0.3282)	—	↓ ↓ ↓ (0.2402)
Left Ab pnd 21	— (0.0137)	—	↓ ↓ ↓ (0.0099)	↓ ↓ ↓ (0.0111)	↓ ↓ ↓ (0.0084)
Left Ab pnd 95	— (0.3563)	—	↓ ↓ (0.3329)	—	↓ ↓ ↓ (0.2548)
Left Ajpnd 21	— (0.0130)	—	↓ ↓ ↓ (0.0103)	↓ ↓ ↓ (0.0108)	↓ ↓ ↓ (0.0092)
Left Ajpnd 95	— (0.3617)	—	↓ (0.3282)	—	↓ ↓ ↓ (0.2543)
Cauda Epididymis					
Right Ab pnd 21	— (0.0099)	—	↓ ↓ ↓ (0.0069)	—	↓ ↓ ↓ (0.0062)
Right Ab pnd 95	— (0.2857)	↓ (0.2719)	↓ ↓ ↓ (0.2309)	—	↓ ↓ ↓ (0.1128)
Right Ajpnd 21	— (0.0097)	—	↓ ↓ ↓ (0.0072)	—	↓ ↓ ↓ (0.0069)
Right Ajpnd 95	— (0.2854)	↓ (0.2716)	↓ ↓ ↓ (0.2316)	↓ (0.2735)	↓ ↓ ↓ (0.1143)
Left Ab pnd 21	— (0.0096)	—	↓ ↓ ↓ (0.0076)	—	↓ ↓ ↓ (0.0064)
Left Ab pnd 95	— (0.2720)	—	↓ ↓ ↓ (0.2263)	—	↓ ↓ ↓ (0.1362)
Left Ajpnd 21	— (0.0095)	—	↓ ↓ ↓ (0.0079)	—	↓ ↓ ↓ (0.0070)
Left Ajpnd 95	— (0.2714)	—	↓ ↓ ↓ (0.2277)	—	↓ ↓ ↓ (0.1363)
Seminal vesicles plus coagulating glands					
Ab pnd 21	— (0.0180)	↓ ↓ ↓ (0.0145)	↓ ↓ ↓ (0.0082)	—	↓ ↓ ↓ (0.0097)
Ab pnd 95	— (1.4707)	—	↓ ↓ ↓ (0.7097)	—	↓ ↓ ↓ (1.1111)
Ajpnd 21	— (0.0178)	↓ ↓ ↓ (0.0143)	↓ ↓ ↓ (0.0085)	—	↓ ↓ ↓ (0.0102)
Ajpnd 95	— (1.4585)	—	↓ ↓ ↓ (0.7378)	—	↓ ↓ ↓ (1.1186)
Prostate					
Whole Gland					
Ab pnd 21	— (0.0483)	↓ (0.0441)	↓ ↓ ↓ (0.0235)	—	↓ ↓ ↓ (0.0292)

(Continued)

F1 Male Offspring Reproductive System Malformations at the Pnd 95 Necropsy[a]

Parameter		Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
		0	50	100	100	500
pnd 95	Ab	— (1.0810)	—	↓↓↓ (0.4226)	—	↓↓↓ (0.8601)
pnd 21	Aj	— (0.0478)	↓ (0.0436)	↓↓↓ (0.0249)	—	↓↓↓ (0.0322)
pnd 95	Aj	— (1.0799)	—	↓↓↓ (0.4261)	—	↓↓↓ (0.8689)
Ventral lobe						
pnd 21	Ab	— (0.0251)	↓ (0.0225)	↓↓↓ (0.0117)	—	↓↓↓ (0.0163)
pnd 95	Ab	— (0.6418)	—	↓↓↓ (0.1897)	—	↓↓↓ (0.4762)
pnd 21	Aj	— (0.0246)	↓ (0.0220)	↓↓↓ (0.0127)	—	↓↓↓ (0.0178)
pnd 95	Aj	— (0.6417)	—	↓↓↓ (0.1899)	—	↓↓↓ (0.4812)
Dorsal lobe						
pnd 21	Ab	— (0.0232)	—	↓↓↓ (0.0118)	—	↓↓↓ (0.0126)
pnd 95	Ab	— (0.4393)	—	↓↓↓ (0.2303)	—	↓↓ (0.3791)
pnd 21	Aj	— (0.0229)	—	↓↓↓ (0.0126)	—	↓↓↓ (0.0139)
pnd 95	Aj	— (0.4381)	—	↓↓↓ (0.2337)	—	↓ (0.3829)
LABC						
pnd 21	Ab	— (0.0562)	↓↓ (0.0474)	↓↓↓ (0.0313)	—	↓↓↓ (0.0419)
pnd 95	Ab	— (1.3577)	↓↓↓ (1.1463)	↓↓↓ (0.5539)	—	↓↓↓ (0.9411)
pnd 21	Aj	— (0.0551)	↓↓ (0.0459)	↓↓↓ (0.0337)	—	↓↓ (0.0456)
pnd 95	Aj	— (1.3508)	↓↓↓ (1.1400)	↓↓↓ (0.5703)	—	↓↓↓ (0.9547)
Cowper's glands						
pnd 21	Ab	— (0.0036)	—	↓↓ (0.0021)	↓ (0.0030)	↓↓ (0.0027)
pnd 95	Ab	— (0.1480)	—	↓↓↓ (0.0688)	—	—
pnd 21	Aj	— (0.0035)	—	—	↓ (0.0030)	↓ (0.0029)
pnd 95	Aj	— (0.1479)	—	↓↓↓ (0.0697)	—	—

(Continued

Text Table 8 (continued)

[a] The control values are presented (in parentheses) for comparison with the values from the treated groups; if there is a statistically significant change, the degree of significance is presented by up/down arrows and the value is in parentheses.
pnd 21 organ weights in grams from Summary Table 13
pnd 95 organ weights in grams from Summary Table 20
Ab = Absolute organ weight in grams
Aj = Adjusted organ weight in grams (adjusted for body weight as covariate).
LABC = Levator ani bulbocavernosus (LABC) muscle

↓, ↓↓, ↓↓↓, statistically significantly reduced at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, by appropriate statistical tests (see summary tables and text for details).

↑, statistically significantly increased at $p < 0.05$ by appropriate statistical tests (see summary tables and text for details).
—, no statistically significant difference from the control group value.

In addition to addressing the specific objectives stated above,

the data in this study invite comparison of the effects of these antiandrogens that work through different mechanisms

Comparison of the Kinds and Incidences of F1 Male Reproductive Malformations by Chemical and by Dose Observed on pnd 95[a]

	VIN Dose		DBP Dose	
Finding	Low	High	Low	High
Cowpers gland missing/reduced in size	##	##	–	##
Epididymides, missing/reduced in size	–	##	–	##
LABC[b] missing/reduced in size/malformed	–	#	–	#
Hypospadias	##	###	–	##
Cleft phallus	##	###	#	##
Prostate: Dorsal	#	##	#	##
Ventral	#	###	–	##
Seminal vesicles, missing/misshapen	#	###	–	##
Epispadias	#	##	–	–
Vaginal pouch	#	##	--	–
Testes undescended	#	##	–	##
Testes embedded in abdominal wall	–	##	–	#
Testes reduced in size	#	##	–	##
Testes flaccid/soft	–	#	–	##
Glans penis not completely detached	#	##	–	##
Males with ≥ 1 cranial suspensory ligament	–	#	–	##

– = no incidence
= small incidence (1-5%), ## = mid range incidence (6-74%), ### = high incidence (>75%)
[a] PND 95 data taken from Individual Animal Tables 20 and 21.
[b] LABC = Levator Ani plus Bulbocavernosus Complex

Would effects observed on pnd 95 have been observed if we only examined one adult male per litter in each group?

CONCLUSIONS

Specific male offspring malformations were detected on pnd 95 but not on pnd 21:

- prostate dorsal lobe abnormal/reduced in size (VIN, both doses; DBP, high dose)
- prostate ventral lobe abnormal/reduced in size (both compounds, both doses)
- epispadias (VIN, both doses)

The incidence of specific male offspring malformations detected on pnd 95 was higher than the incidence of the same malformation observed on pnd 21:

- agenesis of all or parts of the epididymis(des) (high dose of both VIN and DBP)
- hypospadias (low dose VIN)
- missing/reduced in size/abnormal seminal vesicles (high dose of both VIN and DBP)

The effects of VIN on the incidence of hypospadias and ventral prostate agenesis were more obvious at pnd 95 than at pnd 21. This effect was more apparent at the low dose than at the high dose.

- Hypospadias was observed in 9.7% vs 15.8% of the animals on pnd 21 and 95, respectively.
- High dose animals exhibited hypospadias at 80.0% vs 98.6% on pnd 21 and 95, respectively.

The effects of DBP (high dose) on the incidence of epididymal agenesis on pnd 95 was approximately twice that observed on pnd 21, and thus were more obvious on pnd 95 than on pnd 21.

Adverse effects on the weights of some male reproductive tissues were more apparent at pnd 95 than on pnd 21:

- adjusted right or left testis weight (high dose VIN)
- absolute right cauda epididymis weight (low dose VIN)
- adjusted right cauda epididymis weight (low dose VIN and DBP)
- absolute LABC weight (low dose VIN), adjusted LABC weight (high dose VIN and DBP)
- absolute and adjusted Cowper's gland weight (high dose VIN)

CONCLUSIONS (cont'd)

- Adverse reproductive system effects *in toto* (structural malformations and other abnormalities) of the low and high doses of VIN and the high dose of DBP on F1 adult male offspring would most likely be statistically significant with either one or three adult males/litter, and would have been detected with either study design.

- Adverse reproductive system structural effects *in toto* at the low dose of DBP on F1 adult male offspring were clearly biologically significant but not necessarily or likely statistically significant, with either one or three adult males/litter, and provide an example of effects that would not likely be detected with either study design.

- The more males examined per litter, the better the characterization of the litter as responding or not responding adversely to exposure, and the smaller the variance term for pooled litters within each treatment group. The enhanced sensitivity with more males examined per litter would increase the likelihood of detection of effects as statistically and biologically significant.

- Also, for effects with low incidence, such as in the low dose DBP group in this study, the risk with fewer males examined per litter is that the effect might be missed, i.e., the litter would be designated as not responding, on the basis of the one male examined, if that male did not exhibit the effect.

ACKNOWLEDGEMENTS

This study was performed under a subcontract to Battelle Laboratories, Columbus, Ohio for prime contract No. 68-W-01-023 for the U.S. Environmental Protection Agency

Laboratory Staff (RTI International)

- Ms. M.C. Marr (Laboratory Supervisor)
- Ms. C.B. Myers (Reproductive Toxicity Study Supervisor and Data Analyst)
- Mr. W.P. Ross
- Ms. M.C. Rieth
- Ms. V.I. Wilson
- Ms. L.B. Pelletier
- Ms. M.P. Gower
- Ms. N.M. Kuney
- Ms. R.T. Krebs
- Ms. S.W. Pearce
- Ms. K.D. Vick
- Ms. L. McDonald
- Ms. A.J. Parham
- Mr. M.D. Crews
- Mr. C.G. Leach
- Ms. A. Goodman
- Ms. D.B. Bynum

Chemistry

- Dr. E.A. Crecelius, PNNL, Battelle Marine Sciences Laboratory, Sequim, WA.
- Mr. M.M. Veselica, Supervisor, RTI Materials Handling Facility
- Mr. D.L. Hubbard
- Mr. R.A. Price

Animal Care (RTI International)

- Dr. D.B. Feldman, DVM, ACLAM, Veterinarian
- Mr. F.N. Ali, Manager of RTI Animal Research Facility

Quality Assurance

- Ms. D.J. Smith (RTI)
- Ms. M.D. Phillips (RTI)
- Ms. T.M. Kenney (RTI)
- Ms. C. Sexsmith, QA Consultant
- Ms. K. Cummings (ILS)
- Ms. T. Pollock (Battelle)

REFERENCES

Anderson, S., S. Pearce, P. Fail, B. McTaggart, R. Tyl, and L.E. Gray, Jr. (1995). Testicular and adrenal response in adult Long-Evans Hooded rats after antiandrogenic vinclozolin exposure. *J. Andrology* **16**, 43.

Cater, B.R., M.W. Cook, S.D. Gangolli, and P. Grasso (1977). Studies on dibutyl phthalate-induced testicular atrophy in the rat: effect on zinc metabolism. *Toxicol. Appl. Pharmacol.* **41**, 609-618.

Ema, M., H. Amano, T. Itami, and H. Kawasaki (1993). Teratogenic evaluation of di-n-butyl phthalate in rats. *Toxicol. Lett.* **69**, 197-203.

Ema, M., H. Amano, and Y. Ogawa (1994). Characterization of the developmental toxicity of di-n-butyl phthalate in rats. *Toxicology* **86**, 163-174.

Ema, M., R. Kurosaka, H. Amano, and Y. Ogawa (1995a). Comparative developmental toxicity of n-butyl benzyl phthalate and di-n-butyl phthalate in rats. *Arch. Environ. Contam. Toxicol.* **28**, 223-228.

Ema, M., R. Kurosaka, H. Amano, and Y. Ogawa (1995b). Developmental toxicity evaluation of mono-n-butyl phthalate in rats. *Toxicol. Lett.* **78**, 101-106.

Ema, M., E. Miyawaki, and K. Kawashima (1998). Further evaluation of developmental toxicity of di-n-butyl phthalate following administration during late pregnancy in rats. *Toxicol. Lett.* 87-93.

Foster, P.M.D., R.C. Cattley, and E. Mylchreest (2000). Effects of di-n-butyl Phthalate (DBP) on male reproductive development in the rat: Implications for human risk assessment. *Food Chem. Toxicol.* **38**, S97-S99.

Gray, L.E., Jr., J. Ostby, J. Furr, M. Price, D.N.R. Veeramachaneni, and L. Parks (2000). Perinatal exposure to the phthalates DEHP, BBP and DINP, but not DEP, DMP or DOTP alters sexual differentiation of the male rat. *Toxicol. Sci*. **58(2)**, 350-365.

Gray, L.E., Jr. J.S. Ostby, E. Mylchreest, and P.M. Foster (1998). Dibutyl phthalate (DBP) induces antiandrogenic but not estrogenic in vivo effects in LE hooded rats. *Toxicologist* **42(1-S)**, 176.

Gray, L.E., Jr., J.S. Ostby, and W.R. Kelce (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rats. *Toxicol. Appl. Pharmacol.* **129(1)**, 46-52.

Gray, L.E., Jr., J. Ostby, E. Monosson, and W.R. Kelce (1999). Environmental antriandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol. Ind. Health*, **15(1-2)**, 48-64.

Hellwig, J., B. van Ravenzwaay, M. Mayer, and C. Gembardt (2000). Pre- and postnatal oral toxicity of vinclozolin in Wistar and Long-Evans rats. *Regul. Tox. Pharm*. **32(1)**, 42-50.

Kelce, W.R., E. Monosson, and L.E. Gray, Jr. (1994a). In vitro/in vivo evidence that vinclozolin (V) is an environmental antiandrogen. *Biol. Reprod*. **50 (Suppl. 1)**, 189.

Kelce, W.R. E. Monosson, M.P. Gamcik, S.C. Laws, and L.E. Gray, Jr. (1994b). Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol. Appl. Pharmacol.* **126**, 275-285.

Kelce, W.R., C. Lambright, L.E. Gray, Jr., and K. Roberts (1997). Vinclozolin and pp'-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor mediated mechanism. *Toxicol. Appl. Pharmacol*. **142**, 192-200.

Mylchreest, E., R.C. Cattley, M. Sar, and P.M. Foster (1998a). The effects of di(n-butyl) phthalate on prenatal androgen-regulated male sexual differentiation are not mediated by direct interaction with the androgen receptor. *Teratology* **57(4/5)**, 199 (Abstract No. 25).

Mylchreest, E., R.C. Cattley, and P.M. Foster (1998b). Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogen mechanism? *Toxicol. Sci*. **43(1)**, 47-60.

Mylchreest, E., M. Sar, R.C. Cattley, and P.M. Foster (1999). Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol*. **156**, 81-95.

Saillenfait, A.M., J.P. Payan, J.P. Fabry, D. Beydon, I. Langonne, F. Gallissot, and J.P. Sabate (1998). Assessment of the developmental toxicity, metabolism, and placental transfer of di-n-butyl phthalate administered to pregnant rats. *Toxicol. Sci*. **45**, 212-224.

U.S. EPA (1998). U.S. Environmental Protection Agency, Prevention, Pesticides and Toxic Substances. Health Effects Test Guidelines, OPPTS 870.3700, Prenatal Developmental Toxicity Study, EPA 712-C-98-207. August 1998.

U.S. EPA (2000). U.S. Environmental Protection Agency, Prevention, Pesticides and Toxic Substances. Reregistration Eligibility Decision, Vinclozolin, EPA738-R-00-023, October, 2000.

van Ravenzwaay, B. (1992). Discussion of prenatal and reproductive toxicity of Reg. No. 83-258 (vinclozolin). Data submission to USEPA from BASF Corporation, MRID 425813-02.

Wong, C.-I., W.R. Kelce, M. Sar, and E.M. Wilson (1995). Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J. Biol. Chem*. **270**, 19998-20003.5.